VERIFICATION OF TRANSLATION

I, Takashi Fujita, registered Patent Attorney, having my business place at Fukuoka Building, 9th Floor 8-7, Yaesu 2-Chome, Chuo-ku, Tokyo 104-0028 Japan, do hereby declare that I am conversant in the Japanese and the English language and that I am the translator of the documents attached and certify that to the best of my knowledge and belief the following is a true and correct English translation of the specification contained in the Japanese Patent Application No. 1999-236597.

Signature : ______________________

Takashi Fujita

This 7th day of October, 2005

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[TITLE OF THE INVENTION] Screening method

[CLAIMS]

[Claim 1] A method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein, comprising:

(i) measuring cell-stimulating activity when test compound is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when test compound is brought in contact with cells which are not capable of expressing the orphan receptor protein or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of the compounds having agonist activity,

(iii) ① comparing cell-stimulating activity when a ligand candidate compound which is selected by considering the common structure of said compounds having agonist activity is brought in contact with said cells capable of expressing the orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when test compound is brought in contact with said cells capable of expressing the orphan receptor protein or its cell membrane fractions, and ② measuring amount of specific binding between said orphan receptor protein and a candidate compound which promotes or inhibits the function of the orphan receptor protein.

[Claim 2] A compound or a salt thereof obtainable by the screening method according to claim 1.

[Claim 3] A method of screening according to claim 1, wherein said orphan receptor protein is GHS receptor protein.

[Claim 4] A method of screening according to claim 1, wherein said orphan receptor protein is GHS receptor protein, and the ligand candidate compound having the

common structure is a peptide having RF-amide structure at the C-terminus.

[Claim 5] A method of identifying a ligand or its subtypes, comprising:

(i) measuring cell-stimulating activity when test compound is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when test compound is brought in contact with cells which are not capable of expressing the orphan receptor protein or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of compounds having agonist activity, and

(iii) measuring amount of specific binding of the ligand candidate compound having the common structure to said orphan receptor protein.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[FIELD OF THE INVENTION]

The present invention relates to a method of efficiently screening a compound which promotes or inhibits a function of an orphan receptor protein by screening a highly concentrated test compound using activation of cells capable of expressing an orphan receptor as an index and using a common structure of test compounds having a function of an agonist, and also relates to a method of identifying (endogenous) ligands of the orphan protein receptor using the common structure.

[0002]

[BACKGROUND ART]

Physiologically active substances such as various hormones and neurotransmitters regulate various biological functions via receptor proteins present on cell surface. It has been an important means for the

pharmaceutical research and development to search substances which fills, increases and inhibits the function of physiological activity. It is quite important to understand properties of receptor molecules, which work as a point of action in the process. In recent years, sharing the benefit from the development of methodologies in molecular biology, it became possible to analyze receptors for physiological active substances at a molecular level. Among such receptor molecules, a series of receptors possess a common structure containing seven transmembrane domains are known and are thus called seven-transmembrane receptors (7TMR). These receptor proteins are coupled with intracellular signal transduction system through a GTP-binding protein (G protein), and thus are also referred to as G-protein coupled receptors. Seven-transmembrane receptor ligands include various things such as proteins, peptides, amines, amino acids, nucleotides, nucleosides, eicosanoids, phospholipids, scent substances, light and etc.

In recent years, because of the development of analysis technologies for genomes or cDNAs, many genes encoding receptors have been reported. Some of these genes are estimated to belong to the seven-transmembrane receptor family in view of their characteristics of the sequence. However, since the corresponding ligands have not been known yet, they are called an orphan receptor. If a ligand of an orphan receptor can be identified, it is expected to clarify novel physiological functions or pathologies modulated by the receptor and the ligand. Therefore, screening of orphan receptor ligands is considered to be an important means for discovering a new target in pharmaceutical development. There is such an example that orphanin FQ/nociceptin (Meunier, J. -C. Nature 393:211-212,1998) was successfully identified as an endogenous ligand, by examining known ligands or related substances for those orphan receptor showing, to some

extent, remarkable similarities to the receptors for which their ligands are known. However, there is limitation in estimating a structure of ligand only from the structural similarities to known ligands or related substances. In most of cases, an activation of signal transmitters of cells capable of expressing orphan receptor proteins as an index, an identification of endogenous ligands was taking place by purification (Sakurai. T. et al. Cell 92: 573-585,1998; Hinuma, S, et al. Nature 393:272-276,1998; Tatemoto, K. et al. Biochem. Biophys. Res. Commun. 251: 471-476,1998). However, the signal transmission system via a seven-transmembrane receptor is not a single one. Thus, to detect activities derived from endogenous ligands, it is necessary to conduct many assay lines for screening in parallel. The seven-transmembrane receptor ligands include variety of things. It is not easy to select samples rationally for ligand candidates to be tested in assays.

[0003]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

To establish an efficient and reliable method for screening compounds and their salts, which promote or inhibit a function of an orphan receptor protein or a method of identifying (endogenous) ligands of orphan receptor proteins, is considered to be the major issue.

[0004]

[MEANS FOR SOLVING THE PROBLEMS]

In order to solve above problems, the present inventors have made extensive studies, and as a result, found that screening of a compound which promotes or inhibits a function of an orphan receptor protein or its salt can be effectively carried out by (i) measuring cell-stimulating activity when a test compound (preferably, highly concentrated) is brought in contact with cells capable of expressing an orphan receptor protein or its cell membranes fractions, and cell-stimulating activity when a test compound is brought in contact with expression cells

which are not capable of expressing an orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of test compounds having agonist activity, and

(iii) ① comparing cell-stimulating activity when a ligand candidate having said common structure is brought in contact with said cells capable of expressing an orphan receptor protein or its cell membrane fractions with cell-stimulating activity when a candidate compound substance which promotes or inhibits the function of said orphan receptor protein is brought in contact with said cells capable of expressing an orphan receptor protein or its cell membrane fractions, and ② measuring the amount of specific binding between said orphan receptor protein and a compound which promotes or inhibits the function of said orphan receptor protein. Based on these findings, the present inventors made continued extensive studies and as a result, accomplished the present invention.

[0005]

Thus, the present invention relates to, for example, the following:

(1) A method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein, comprising:

(i) measuring cell-stimulating activity when a test compound is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when the test compound is brought in contact with cells which are not capable of expressing the orphan receptor protein or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of test compound(s) having agonist activity,

(iii) ① comparing cell-stimulating activity when a ligand candidate compound having the common structure is brought in contact with said cells capable of expressing the orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when a candidate compound which promotes or inhibits the function of the orphan receptor protein is brought in contact with said cells capable of expressing the orphan receptor protein or its cell membrane fractions, and ② measuring the amount of specific binding between said orphan receptor protein and the candidate compound which promotes or inhibits the function of the orphan receptor protein, (2) A compound or a salt thereof obtainable by the screening method according to the screening method described in above (1),

(3) A method of screening according to the above (1), wherein said orphan receptor protein is GHS receptor protein,

(4) A method of screening according to the above (1), wherein said orphan receptor protein is GHS receptor protein, and the ligand candidate compound having the common structure is a peptide having RF-amide structure at the C-terminus, and

(5) A method of identifying a ligand or its subtypes, comprising:

(i) measuring cell-stimulating activity when test compound is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions, and cell-stimulating activity when test compound is brought in contact with cells which are not capable of expressing the orphan receptor protein or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of compounds having agonist activity, and

(iii) measuring amount of specific binding of the ligand candidate compound having the common structure to said orphan receptor protein.

[0006]

[EMBODIMENTS OF THE INVENTION]

(A) The orphan receptor protein:

In this specification, "orphan receptor proteins" means proteins the ligand of which has not been known, including the both publicly known and unknown ones.

Examples of orphan receptor proteins are; GHS receptor protein (Howard, A. D. et al., Science, 273: 974-977, 1996) used in Example 1 described later, FM-3 receptor protein (Tan, C. P. et al, Genomics 52,223-229, 1998), mas receptor protein (Young D. et. al., Proc. Natl. Acad. Sci. USA, 85,5339-5342, 1988), etc. Moreover, they are listed in the Swiss-plot database for orphan receptors.

The orphan receptor proteins useful in the present invention may form salts. The salts of "orphan receptor proteins" may be salts with physiologically acceptable bases (e.g., alkali metals), or salts with physiologically acceptable acids (organic acids, inorganic acids), especially physiologically acceptable acid addition salts. Examples of such salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

The DNA encoding an orphan receptor protein used in the present invention may be any DNA comprising DNA encoding an orphan receptor protein. It could be a genome DNA, genome DNA library, a cDNA derived from any cells of human and other warm-blooded animals (e.g., guinea pig, rat, mouse, rabbit, swine, sheep, bovine, monkey, etc.) such as retina cells, liver cells, splenocytes, nerve cells, glial

cells, □ cells of pancreas, bone marrow cells, mesangial cells, Langerhans' cells, epidermic cells, epithelial cells, endothelial cells, fibroblasts, fibrocytes, myocytes, fat cells, immune cells (e.g., macrophage, T cells, B cells, natural killer cells, mast cells, neutrophil, basophil, eosinophil, monocyte), megakaryocyte, synovial cells, chondrocytes, bone cells, osteoblasts, osteoclasts, mammary gland cells, hepatocytes or interstitial cells, the corresponding precursor cells, stem cells, cancer cells, etc., or any tissues where such cells are present, e.g., brain or any region of the brain (e.g., retina, olfactory bulb, amygdaloid nucleus, basal ganglia, hippocampus, thalamus, hypothalamus, subthalamic nucleus, cerebral cortex, medulla oblongata, cerebellum), spinal cord, grandula pituitaria, hypophysis, stomach, pancreas, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract (e.g., large intestine and small intestine), blood vessel, heart, thymus, spleen, submandibular gland, peripheral blood, prostate, testis, ovary, placenta, uterus, bone, joint, skeletal muscle, hemocyte type cells or its cultured cell (e.g., MEL, M1, CTLL-2, HT-2, WEHI-3, HL-60, JOSK-1, K562, ML-1, MOLT-3, MOLT-4, MOLT-10, CCRF-CEM, TALL-1, Jurkat, CCRT-HSB-2, KE-37,SKW-3, HUT-78, HUT-102, H9, U-937, THP-1,HEL,JK-1, CMK, KO-812, MEG-01 etc.). The DNA encoding an orphan receptor protein may also be a cDNA library derived from said tissues/cells, or a synthetic DNA. The vector used for the library may be any of bacteriophage, plasmid, cosmid and phagemid. The DNA may also be directly amplified by reverse transcriptase polymerase chain reaction (hereinafter abbreviated as RT-PCR) using the total RNA or mRNA fraction prepared from the cells and tissues.

[0007]

The DNA encoding an orphan receptor protein can be prepared according to the following genetic engineering method.

For cloning of the DNA that completely encodes the orphan receptor protein, the DNA of interest may be either amplified from the above-described DNA library and the like by PCR using synthetic DNA primers containing a part of the base sequence of the orphan receptor protein, or the DNA inserted into an appropriate vector can be selected by hybridization with a labeled DNA fragment or synthetic DNA that encodes a part or entire region of the orphan receptor protein of the present invention. The hybridization can be carried out, for example, according to the method described in Molecular Cloning, 2nd, J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989. The hybridization may also be performed using commercially available library in accordance with the protocol described in the attached instructions.

The cloned DNA encoding an orphan receptor protein can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may contain ATG as a translation initiation codon at the 5' end thereof and may further contain TAA, TGA or TAG as a translation termination codon at the 3' end thereof. These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

[0008]

(B) The cells capable of expressing an orphan receptor protein and its cell membrane fractions, or the cells which do not express orphan receptor protein and its cell membrane fractions:

In this specification, "cells capable of expressing an orphan receptor protein" means host cells expressing the above orphan receptor protein (A).

As to the method of expressing an orphan receptor protein at a host cell, it can be expressed using the DNA encoding the above orphan receptor protein (A) according to the following method.

That is, the expression vector for the receptor protein can be prepared, for example, by (a) excising the desired DNA fragment from the DNA encoding the receptor protein of the present invention, and then (b) ligating the DNA fragment with an appropriate expression vector downstream a promoter in the vector.

Examples of the vector include plasmids derived form E. coli (e.g., pBR322, pBR325, pUC12, pUC13), plasmids derived from Bacillus subtilis (e.g., pUB110, pTP5, pC194), plasmids derived from yeast (e.g., pSH19, pSH15), bacteriophages such as □ phage, etc., animal viruses such as retrovirus, vaccinia virus, baculovirus, etc. The promoter used in the present invention may be any promoter if it matches well with a host to be used for gene expression.

[0009]

In the case of using animal cells as the host for transformation, examples of the promoter include SV40-derived promoter, retrovirus promoter, metallothionein promoter, heat shock promoter, a cytomegalovirus promoter, SR□ promoter, etc. Where the host is bacteria of the genus Escherichia, preferred examples of the promoter include Trp promoter, T-7 promoter, lac promoter, recA promoter, □PL promoter, lpp promoter, etc. In the case of using bacteria of the genus Bacillus as the host, preferred examples of the promoter are SPO1 promoter, SPO2 promoter and penP promoter, etc.. When yeast is used as the host, preferred examples of the promoter are PHO5 promoter, PGK promoter, GAP promoter, ADH1 promoter, GAL promoter, etc.. When insect cells are used as the host, preferred examples of the promoter include polyhedrin prompter and P10 promoter, etc.. In addition, in the present invention, to measure cell-stimulating activity

mediated by an orphan receptor protein, preferred hosts are animal cells and insect cells.

In addition, if desired, the expression vector may further optionally contain an enhancer, a splicing signal, a poly A addition signal, a selection marker, SV40 replication origin (hereinafter sometimes abbreviated as SV40ori) etc. Examples of the selection marker include dihydrofolate reductase (hereinafter sometimes abbreviated as dhfr) gene [methotrexate (MTX) resistance], ampicillin resistant gene (hereinafter sometimes abbreviated as Amp^r), neomycin resistant gene (hereinafter sometimes abbreviated as Neo^r, G418 resistance), etc. In particular, when DHFR gene is used as the selection marker in CHO ($dhfr^-$) cells, selection can also be made on thymidine free media.

If necessary and desired, a signal sequence that matches with a host is added to the N-terminus of the receptor protein of the present invention. Examples of the signal sequence that can be used are Pho A signal sequence, OmpA signal sequence, etc. in the case of using bacteria of the genus Escherichia as the host; □-amylase signal sequence, subtilisin signal sequence, etc. in the case of using bacteria of the genus Bacillus as the host; mating factor (MF□) signal sequence, invertase signal sequence, etc. in the case of using yeast as the host; and insulin signal sequence, □-interferon signal sequence, antibody molecule signal sequence, etc. in the case of using animal cells as the host, respectively.

Using the vector containing the DNA encoding an orphan receptor protein thus constructed, transformants can be manufactured.

[0010]

Examples of the host, which may be employed, are bacteria belonging to the genus Escherichia, bacteria belonging to the genus Bacillus, yeast, insect cells,

insects and animal cells, etc. As mentioned above, preferred are insect cells and animal cells.

Specific examples of the bacteria belonging to the genus Escherichia include Escherichia coli K12 DH1 (Proc. Natl. Acad. Sci. U.S.A., 60, 160 (1968)), JM103 (Nucleic Acids Research, 9, 309 (1981)), JA221 (Journal of Molecular Biology, 120, 517 (1978)), HB101 (Journal of Molecular Biology, 41, 459 (1969)), C600 (Genetics, 39, 440 (1954)), etc.

Examples of the bacteria belonging to the genus Bacillus include Bacillus subtilis MI114 (Gene, 24, 255 (1983)), 207-21 (Journal of Biochemistry, 95, 87 (1984)), etc.

[0011]

Examples of yeast include Saccharomyces cereviseae AH22, AH22R$^-$, NA87-11A, DKD-5D, 20B-12, etc.

As the insect, for example, a larva of Bombyx mori can be used (Maeda, et al., Nature, 315, 592 (1985)).

As the insect cells, for example, for the virus AcNPV, Spodoptera frugiperda cells (Sf cells), MG1 cells derived from mid-intestine of Trichoplusia ni, High Five™ cells derived from egg of Trichoplusia ni, cells derived from Mamestra brassicae, cells derived from Estigmena acrea, etc.; and for the virus BmNPV, Bombyx mori N cells (BmN cells), etc. are used. Examples of the Sf cell which can be used are Sf9 cells (ATCC CRL1711) and Sf21 cells (both cells are described in Vaughn, J. L. et al., In Vivo, 13, 213-217 (1977).

Examples of animal cells include monkey cells COS-7, Vero, Chinese hamster cells CHO , dhfr gene deficient Chinese hamster cells CHO (hereinafter simply referred to as CHO(dhfr$^-$) cell), mouse L cells, mouse 3T3, mouse myeloma cells, human HEK293 cells, human FL cells, C127 cells, BALB3T3cell, Sp-2/O cells) etc.

Bacteria belonging to the genus Escherichia can be transformed, for example, by the method described in Proc.

Natl. Acad. Sci. U.S.A., 69, 2110 (1972) or Gene, 17, 107 (1982). Bacteria belonging to the genus Bacillus can be transformed, for example, by the method described in Molecular & General Genetics, 168, 111 (1979).

Yeast can be transformed, for example, by the method described in Proc. Natl. Acad. Sci. U.S.A., 75, 1929 (1978), etc.

[0012]

Insect cells or insects can be transformed, for example, according to the method described in Bio/Technology, 6, 47-55(1988), etc.

Animal cells can be transformed, for example, according to the method described in Virology, 52, 456 (1973).

The method of introduction to the cell of orphan receptor protein expression vector, for example, lipofection (Felgner, P.L. et al. Proc. Natl. Acad. Sci. U.S.A., 84, 7413 (1987)), Calcium phosphate (Graham, F.L. and van der Eb, A.J. Virology, 52, 456-467 (1973)), electroporation (Nuemann, E. et al. EMBO J., 1, 841-845 (1982)) are listed.

The cells capable of expressing an orphan receptor protein can be obtained using such methods.

Furthermore, as to the method of stably expressing an orphan receptor protein using animal cells, there is a method in which the above animal cells, into which the expression vector has been introduced and the vector has been inserted into the chromosome, are selected by clonal selection. To be more specific, using the above selection marker as an index, a transformant can be selected. For those animal cells obtained by use of the selection marker, it is possible to obtain stable animal cell lines capable of highly expressing the orphan receptor protein by repeating the clonal selection. Moreover, using dhfr gene as a selection marker, by culturing the cells with gradually increasing the concentration of MTX, and selecting the cell

only from resistant cell lines, it is possible to obtain highly expression animal cell lines by amplifying the DNA encoding orphan receptor protein in the cell with dhfr gene.

Where the host is bacteria belonging to the genus Escherichia or the genus Bacillus, the transformant can be appropriately incubated in a liquid medium which contains materials required for growth of the transformant such as carbon sources, nitrogen sources, inorganic materials, and so on. Examples of the carbon sources include glucose, dextrin, soluble starch, sucrose, etc. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, meat extract, soybean cake, potato extract, etc. Examples of the inorganic materials are calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc. In addition, yeast extract, vitamins, growth promoting factors etc. may also be added to the medium. Preferably, pH of the medium is adjusted to about 5 to about 8.

[0013]

A preferred example of the medium for incubation of the bacteria belonging to the genus Escherichia is M9 medium supplemented with glucose and Casamino acids (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972). If necessary and desired, a chemical such as 3□-indolylacrylic acid can be added to the medium thereby to activate the promoter efficiently. Where the bacteria belonging to the genus Escherichia are used as the host, the transformant is usually cultivated at about 15°C to about 43°C for about 3 hours to about 24 hours. If necessary and desired, the culture may be aerated or agitated.

Where the bacteria belonging to the genus Bacillus are used as the host, the transformant is cultivated generally at about 30°C to about 40°C for about 6 hours to about 24 hours. If necessary and desired, the culture can be aerated or agitated.

Where yeast is used as the host, the transformant is cultivated, for example, in Burkholder's minimal medium (Bostian, K. L. et al., Proc. Natl. Acad. Sci. U.S.A., 77, 4505 (1980)) or in SD medium supplemented with 0.5% Casamino acids (Bitter, G. A. et al., Proc. Natl. Acad. Sci. U.S.A., 81, 5330 (1984)). Preferably, pH of the medium is adjusted to about 5 to about 8. In general, the transformant is cultivated at about 20°C to about 35°C for about 24 hours to about 72 hours. If necessary and desired, the culture can be aerated or agitated.

[0014]

Where insect cells or insects are used as the host, the transformant is cultivated in, for example, Grace's Insect Medium (Grace, T. C. C., Nature, 195, 788 (1962)) to which an appropriate additive such as immobilized 10% bovine serum is added. Preferably, pH of the medium is adjusted to about 6.2 to about 6.4. Normally, the transformant is cultivated at about 27°C for about 3 days to about 5 days and, if necessary and desired, the culture can be aerated or agitated.

Where animal cells are employed as the host, the transformant is cultivated in, for example, MEM medium containing about 5% to about 20% fetal bovine serum (Science, 122, 501 (1952)), DMEM medium (Virology, 8, 396 (1959)), RPMI 1640 medium (The Journal of the American Medical Association, 199, 519 (1967)), 199 medium (Proceeding of the Society for the Biological Medicine, 73, 1 (1950)), etc. Preferably, pH of the medium is adjusted to about 6 to about 8. The transformant is usually cultivated at about 30°C to about 40°C for about 15 hours to about 60 hours and, if necessary and desired, the culture can be aerated or agitated.

In the case of using $CHO^{-}(dhfr^{-})$ cells and dhfr gene as a selection marker, DMEM medium containing thymidine-free dialysed fetal bovine serum is preferred to use.

[0015]

The cell membrane fraction refers to a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by fractionation using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in an orphan receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the receptor protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell. As the amount of expression increases, the ligand binding activity per unit of membrane fraction (specific activity) increases so that not only the highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot.

The cells which do not express the orphan receptor proteins or its cell membrane fraction refers to the cells listed above as a host cell, which does not express the orphan receptor proteins. As its cell membrane fraction, those same as above can be used.

[0016]

(C) Test compound:

In this specification, "test compound" refers to natural/non-natural peptides, natural/non-natural proteins, natural/non-natural non-peptide compounds, synthetic compounds, natural/unnatural fermentation products as examples.

Those peptides, proteins, compounds and fermentation products used for test compounds can form salts. For salts of the orphan receptor protein, preferred are salts with physiologically acceptable bases (e.g., alkali metal) or acids (organic acids, inorganic acids), especially physiologically acceptable acid addition salts. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

[0017]

(D) Cell stimulating activity:

When a test compound is brought in contact with cells capable of expressing an orphan receptor protein and when a test compound is brought in contact with cells which are not capable of expressing orphan receptor protein, each cell-stimulating activity can be measured using as index, for example, (a) change in extracellular pH, (b) arachidonic acid release, (c) acetylcholine release, (d) intracellular Ca^{2+} release, (e) change in intracellular cAMP production, (f) change in intracellular cGMP production, (g) inositol phosphate production, (h) change in cell membrane potential, (i) phosphorylation of intracellular proteins, (j) activation of c-fos, (k) binding of GTPγS, (l) expression of a reporter gene by a publicly known method, or using an assay kit commercially available.

Among the above methods, for the measurements of cell-stimulating activity performed in the screening method or ligand identification method according to the present invention, preferred is a measuring method using as an index the change in pH outside a cell.

Specifically, cells expressing an orphan receptor protein or its cell membrane fraction, and cells which do not express the orphan receptor protein or its cell membrane fraction are first cultured on a multi-well plate, respectively. Prior to the measurement of the cell-stimulating activity, the medium is replaced with fresh medium or with an appropriate non-cytotoxic buffer, followed by incubation for a given period of time in the presence of a test compound. Subsequently, the cells are extracted or the supernatant is recovered and the resulting product is quantified by appropriate procedures.

Where it is difficult to detect the production of the index substance forcell-stimulating activity due to a degrading enzyme contained in the cells, an inhibitor against such a degrading enzyme may be added prior to the assay. For detecting activities such as the cAMP production suppression activity, the baseline production in the cells or the cell fractions is increased by forskolin or the like and the suppressing effect on the increased baseline production may then be detected.

To conduct the screening by measuring the cell stimulating activities, it requires appropriate cells capable of expressing an orphan receptor protein or its cell membrane fractions and appropriate cells which do not express orphan receptor protein or its cell membrane fractions. The cells capable of expressing an orphan receptor protein can be either a stable or a transient expression strain.

To measure cell-stimulating activity, it is preferred to keep the concentration as high as possible as long as it is possible to distinguish specific responses

of the cells in order to detect the weak activities of agonists. In this case, "High concentration" means normally 10^{-8}M to 1M, preferably 10^{-6}M to 10^{-2}M. The same compounds described in above (C) may be used as the test compounds.

[0018]

Moreover, the details of the above cell-stimulating activity assay system are described as follows:

(1) Cell-stimulating activity assay system characterized by measuring the fluctuation of extracellular pH (acidification rate)

The cell-stimulating activity can be measured by measuring the extracellular pH change caused by reaction of a test compound having agonist activity with cells capable of expressing an orphan receptor protein or its cell membrane fractions, using a cytosensor (Manufactured by Molecular Divice).

Described below is the detail method of measuring cell-stimulating activity of a test compound by Cytosensor for measuring the extracellular pH changes

① After cultivating cells capable of expressing the orphan receptor protein or its cell membrane fraction for about 2 to 48 hours, preferably about 5 to 24 hours (for example, in the capsule of Cytosensor device), the pH of the medium is stabilized.

Until the pH becomes stable, a medium (e.g. RPMI1640 medium with 0.1% fatal bovine serum (Prepared by Molecular Device)) is perfused.

② Then, a test compound is brought in contact with cells capable of expressing the orphan receptor protein or the orphan receptor protein expressed on its cell membrane fractions.

It is general for such contact to perfuse the medium containing a test compound to cells capable of expressing an orphan receptor protein or its cell membrane fractions.

③ Then, the pH change in the medium when a medium containing a test compound is brought in contact with the cells capable of expressing an orphan receptor protein or an orphan receptor protein expressed on its cell membrane fractions, is measured.

④ Then, the above methods ① to ③ are conducted using the cells which do not express the orphan receptor protein or its cell membrane fractions.

[0019]

(2) Cell-stimulating activity assay system characterized by measuring the radioactivity of GTPγS

When a test compound having agonist activity stimulates cells capable of expressing an orphan receptor protein, an intercellular G-protein becomes active and, as a result, GTP binds thereto. The same phenomena can be observed with the cell membrane fractions capable of expressing the orphan receptor protein. Generally, GTP is converted to GDP by hydrolysis. When GTPγS is added to the reaction solution, GTPγS binds G-protein (same as GTP does), and it keeps binding to the cell membrane containing the G-protein without being hydrolyzed. Using labeled GTP γS, it is possible to measure the receptor expression cell-stimulating activity of the test compound by measuring the radioactivity remaining in the cell membrane. Applying this reaction, a stimulating activity of a test compound with respect to cells capable of expressing an orphan receptor protein and the cells which do not express the orphan receptor protein. This method is an assay using an orphan receptor protein and the cell membrane fractions containing cells which are not capable of expressing the orphan receptor, and is also an assay to measure cell-stimulating activity. In this assay, a substance which shows an activity to promote binding GTPγs to an orphan receptor protein cell membrane, and which shows no activity to promote binding of GTPγS to a cell membrane

which does not express an orphan receptor protein, may be a ligand candidate compound.

The detail description for measuring cell-stimulating activity by this method is as follows.

① A cell membrane fraction containing an orphan receptor protein is diluted with a membrane dilution buffer (e.g. 50mM Tris, 5mM $MgCl_2$,150mM NaCl, 1μM GDP, 0.1% BSA pH7.4).

The dilution rate may vary according to the amount of receptor protein expression.

② The solution obtained from Step ① is transferred partially to an appropriate container (e.g. Falcon 2053). A test compound is added to the container, and then [^{35}S]GTPγS is added to make the final concentration to become 200pM.

③ After the medium obtained from Step ② is kept at 25℃ for an hour, a buffer solution for washing (e.g. ice-cooling 50mM Tris, 5mM $MgCl_2$, 150mM NaCl, 0.1% BSA, 0.05% CHAPS pH7.4 1.5ml) is added. Then, the solution is filtered with, for example, a glass fiber filtering paper GF/F.

④ The filtering paper is kept at 65℃ for 30minutes for drying. Then, the radioactivity of [^{35}S]GTPγS in the membrane fraction left on the filtering paper is measured on a liquid scintillation counter.

⑤ Using a cell membrane fraction which does not contain an orphan receptor protein, the steps ① to ④ are conducted.

[0020]

(3) Cell-stimulating activity measurement assay system characterized by measuring change in intracellular cAMP

Cells capable of expressing an orphan receptor protein change amount of intracellular cAMP by the agonist stimuli of the test compounds containing agonist activity. Using this reaction, the cell stimulating activities of the test

compound against the cells capable of expressing an orphan receptor protein can be measured.

Using the anti-cAMP antibody obtained by immunized mice, rats, rabbits, goats and cows and ^{125}I labeled cAMP (both are commercially available), the amount of cAMP production for the varieties of animal cells expressing an orphan receptor protein can be measured by RIA or other EIA system which utilizes the combination of anti-cAMP antibody and the labeled cAMP.

It is possible to conduct an assay like SPA method using beads containing the scintillant to which an anti-cAMP antibody is fixed using Protein A or an antibody to an animal IgG used for production of an anti-cAMP antibody, and ^{125}I labeled cAMP (for example, using the kit manufactured by Amarsham pharmacia Biotech).

Using this assay system, it is possible to measure cAMP production promoting activity. Also, it is possible to measure cell-stimulating activity (cAMP production inhibition activity) by increasing amount of intercellular cAMP using a substance which causes increase in the amount of intercellular cAMP such as Forskolin, and observing any change in the amount of intracellular cAMP when a test compound is added.

The detail description for measuring cell-stimulating activity by this method is as follows.

① Animal cells (e.g. CHO cell) expressing an orphan receptor protein are placed in a 24-well plate, and cultivated with an appropriate concentration ($5x10^4$ cell/well) for about 48 hours.

② The animal cells expressing the orphan receptor protein are washed with a buffer solution (e.g. Hanks' buffer containing 0.2mM 3-isobutyl-methylxanthine, 0.05% BSA and 20mM HEPES (pH7.4), hereinafter referred to as reaction buffer).

③ An appropriate amount (e.g. about 0.5ml) of the reaction buffer is added to the cell, and kept in an incubator for 30 minutes.

④ Then, the reaction buffer is removed from the system, and an appropriate amount (e.g. about 0.25ml) of fresh reaction buffer is added to the cell. Then, an appropriate amount (e.g. about 0.25ml) of the reaction buffer (to measure the cAMP production inhibition activity, it prefers to use the buffer containing 2 μM forskolin) with an appropriate amount (e.g. 1nM) of the test compound is added to the cell. The solution is reacted at 37℃ for 24 minutes.

⑤ 20% of perchloric acid is added to stop the reaction. Then, by placing it on ice, the intracellular cAMP is extracted.

⑥ The amount of cAMP in the extraction is measured by using cAMP EIA kit (Amasham pharmacia biotech).

⑦ The Steps ① to ⑥ are conducted with the cell which does not express the orphan receptor protein.

[0021]

(4) Cell-stimulating activity assay system characterized by introducing CRE-reporter gene

The DNA containing CRE (cAMP response element) is inserted into the multi-cloning site which is upperstream of luciferase gene of Picagene basic vector or Picagene enhancer vector (Toyo Ink). It named as CRE-reporter gene vector.

In the cell transfected by CRE-reporter gene vector, a stimulus with the increase of cAMP, induces an expression of luciferase gene through CRE and a production of luciferase protein. By measuring the luciferase activity, it is possible to detect the change in the amount of cAMP in CRE-reporter gene vector induction cells.

Cell-stimulating activity can be measured using the cells capable of expressing an orphan receptor protein, to which CRE-reporter gene vector is transfected.

The detailed description for the method of measuring cell-stimulating activity is as follows.

① Cells capable of expressing an orphan receptor to which the CRE-reporter gene is introduced, is placed in a 24-well plate with an appropriate concentration ($5x10^3$ cell/well), and cultivated for about 48 hours.

② The cells are washed with an appropriate amount (e.g. 0.2mM) of buffer solution (e.g. Hanks' buffer containing (pH7.4) 3-isobutyl-methyl xanthine, 0.05% BSA and 20mM HEPES, hereinafter, Hanks' buffer containing (pH7.4) 0.2mM 3-isobutyl-methyl xanthine, 0.05% BSA and 20mM HEPES, is referred to as reaction buffer).

③ An appropriate amount (e.g. about 0.5ml) of the reaction buffer is added to the cells. Then, the mixture is kept warm in a cultivator for 30 minutes.

④ Then, the reaction buffer is removed from the system. An appropriate amount (e.g. about 0.25ml) of the reaction buffer is added to the cells. Then, an appropriate amount (e.g. about 0.25ml) of the reaction buffer (to measure the cAMP production inhibition activity, it prefers to use the buffer containing 2 μM forskolin) and an appropriate amount (e.g.1nM) of the test compounds are added to the cells. The reaction is carried out at 37℃ for 24 hours.

⑤ The cells are dissolved in a cell lysis solution for Picagene (Toyo Ink). To the cell lysate, a luminescent substance (Toyo Ink) is added.

⑥ The luminescence is measured by a luminometer, a liquid scintillation counter, a top counter or the like.

⑦ The steps ① to ⑥ are conducted with the cells which do not express the orphan receptor protein or its cell membrane fraction.

Alkaline phosphatase, chloramphenicol, acetyltransferase or β-galactosidase can be used as a reporter gene, besides luciferase. The activity of the gene production of reporter gene can be measured easily

using commercially available measuring kit. That means, the activity of alkaline phosphatase can be measured by Lumi-Phos 530(Wako); the activity of chloramphenicol and acetyltransferase can be measured by FAST CAT Chrolamphenicol Acetyltransferase Assay Kit(Wako); and the activity of β-galactosidase can be measured by Aurora Gal-XE (Wako).

[0022]

(5) Cell-stimulating activity assay system characterized by measuring arachidonic acid release

The cells capable of expressing an orphan receptor protein release the metabolic substance of arachidonic acid to outside of the cells due to the stimulus by an agonist. If arachidonic acid having radioactivity is taken into the cell beforehand, it is possible to measure cell-stimulating activity by measuring the radioactivity released outside the cells. In this process, if the test compound is added, it is possible to measure cell-stimulating activity by checking the arachidonic acid metabolite release activity of the test compound.

The detail description for the method of measuring cell-stimulating activity is as follows.

① Cells capable of expressing an orphan receptor are placed in a 24-well plate, and cultivated with an appropriate concentration (5×10^4 cell/well) for about 24 hours.

② After cultivation, an appropriate amount (0.25μCi/well) of [^{3}H] arachidonic acid is added. 16 hours after adding [^{3}H] arachidonic acid, the cells are washed with a washing solution (Hanks' buffer (pH7.4), containing 0.05% BSA and 20mM HEPES). Then, the test compound dissolved in a buffer solution (e.g., Hanks' buffer (pH7.4) containing 0.05% BSA and 20mM HEPES, hereinafter, Hanks' buffer (pH7.4) containing 0.05% BSA and 20mM HEPES is referred to as reaction buffer) is added to each well.

③ After incubating at 37℃ for 60 minutes, an appropriate amount of the reaction buffer (e.g.400 μ l) is added to a scintillator. Then, the amount of [H^3] arachidonic acid metabolite is measured by a scintillation counter.

④ The steps ① to ③ are conducted with the cells which do not express an orphan receptor protein.

[0023]

(6) Cell-stimulating activity assay system, which is characterized by measuring intracellular Ca^{2+} release

When a test compound having agonist activity stimulates cells which are expressing an orphan receptor, the intracellular Ca^{2+} concentration increases. Using this fact, the cell-stimulating activity can be measured.

① Cells capable of expressing an orphan receptor protein are placed on a sterilized cover glass for a microscope. After about 2 days, the medium is replaced with HBSS in which an appropriate amount (4mM) of Fura-2 AM (Dojin Kagaku) is suspended, and left for 2 and half hours at room temperature.

② After washing with HBSS, a cover glass to a cuvet is set. The increase in the ratio of intensity of fluorescence at 505nm where the excitation wave length is 340nm and 380nm, is measured by a spectrophotofluorometer when the test compound is added.

FLIPR (Manufactured by Molecular device) can be also used as follows.

That is, ① Fluo-3 AM (Manufactured by Dojin Kagaku) is added to the cell suspension to let the cell comprise Fluo-3AM. The supernatant is washed by centrifuging several times, and then cells are placed in a 96-well plate.

② The cells are set to a FLIPR device, and a test compound is added in the same way as Fura-2. The change in the intensity of the fluorescence observed when the test compound is added, is measured. Also, cell-stimulating activity can be measured by allowing cells capable of expressing an orphan receptor protein to co-express the

gene of a protein such as Aequorin which emits light by the increase of the intercellular calcium, utilizing the fact that the increase of the intercellular calcium ion causes Aequorin to become calcium binding type and it emits light as a result, and measuring the change in the intensity of luminescence observed when the test compound is added. This method is almost same as above except that this method does not require the cell to take in the fluorescence substance.

To measure the change in the intercellular calcium ion concentration more easily, it is possible to use cells which expresses a modified G-protein such as chimera G-protein at the same time. Said chimera G-protein refers to a G-protein in which a G-protein such as Gi, Go, Gs or the like, which does not use Ca^{2+} in a signal transduction system is substituted by the functional domain of G-protein such as G_9, G_{11} or the like, which uses Ca^{2+} in a signal transduction system. By using said chimera protein, the signal transmission of any G-protein can be monitored through Ca^{2+} change.

③ The steps ① to ② are conducted with using cells which are not capable of expressing an orphan receptor protein.

[0024]

(7) Cell-stimulating activity assay system, which is characterized by measuring inositol phosphate production

By adding a test compound having agonist activity to cells capable of expressing an orphan receptor protein, the concentration of inositol triphosphate rises. By observing the reaction for cells capable of expressing orphan receptor protein caused by the test compound, cell-stimulating activity can be measured.

① The detail description for the method of measuring cell-stimulating activity is as follows. Cells capable of expressing an orphan receptor are placed in a 24-well plate, and left for one day, and cultivated for one day

in a medium in which myo-[2-^{3}H]inositol (2.5 μCi/well) is added. After washing cells with the medium, a test compound is added thereto and 10% Perchloric acid is added to stop the reaction.

② The reaction solution is neutralized with an appropriate amount of KOH (e.g. 1.5M) and an appropriate amount of HEPES solution (60mM). The cells are passed through a column filled with AG1x8 resin (Bio Rad). After washing, the radioactivity, which is eluted by an appropriate amount of $HCOONH_4$ (e.g. 1M) and an appropriate amount of HCOOH (e.g. 0.1M) is measured by a liquid scintillation counter.

③ The steps ① to ② are conducted with using cells which are not capable of expressing an orphan receptor protein.

[0025]

(E) Test compounds having agonist activity

In this specification, "a test compound containing agonist activity" means that a test compound described in above (c)(e.g., natural/non-natural peptide, natural/non-natural protein, natural/non-natural non-peptide, synthetic compounds and natural/unnatural fermentation products) or test compound, which shows cell-stimulating activity when it is brought into contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions in any one of the above cell-stimulating activity assay systems (preferably, cell-stimulating activity assay system characterized by measuring the change in the extracellular pH (acidification rate)) and when cell-stimulating activity is not observed in the case of using cells which are not capable of expressing an orphan receptor protein or its cell membrane fractions (e.g. natural/non-natural peptide, natural/non-natural protein, natural/non-natural non-peptide, synthetic compounds and natural/unnatural fermentation products as examples).

More specifically, examples of test compounds having agonist activity when an orphan receptor protein is GHS receptor (Howard et al., Science 273, 974-977, 1996), are peptides containing C-terminus R-F structure, and more specifically, peptides having the amino acid sequence represented by SEQ ID NO:1, 6, 9-10, 13 and 16-20. The peptides, protein, compounds and fermentation product which are test compounds having agonist activity can form salts. These salts can be salts with physiologically acceptable bases (e.g., alkali metal) or acids (organic acids, inorganic acids). Among others, physiologically acceptable acid addition salts are preferred. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

As to the cell-stimulating activity assay systems according to the above (D), the judgment standard to determine whether a test compound is a test compound having agonist activity or not, is described as follows. However, this standard is just an example, and thus it is not used to limit the interpretation as to whether a test compound has agonist activity or not.

[0026]

In the case of using the cell-stimulating activity assay system which characterized by measuring a change in exracellular pH (acidification rate) according to above (D)-(1), when an extracellular pH is not less than 105% when a test compound is brought in contact with a cell (where an extracellular pH before a test compound is brought to be in contact with the cell is set as 100%), and when cell-stimulating activity is not observed in the case of using orphan receptor protein non-expression cell or its

cell membrane fractions, such test compound is selected as the test compound having agonist activity.

In the case of using cell-stimulating activity assay system which is characterized by measuring the radioactivity labeled GTPγS as described in the above (D)-(2), when radio-activation of a test region is not less than 105% when a test compound is added (where radioactivity without adding a test compound is set as 100%) and when cell-stimulating activity is not observed in the case of using cells which do not express the orphan receptor protein or its cell membrane fractions, such compound is selected as a test compound having agonist activity.

In the case of using cell-stimulating activity assay system which characterized by measuring the change in the intracellular cAMP as described in (D)-(3), when the amount of produced cAMP is no more than 95% in the case of adding a test compound (where the amount of produced cAMP by forskolin stimulation is set as 100% in the case of using the cAMP production inhibition function as an index), and when cell-stimulating activity is not observed in the case of using orphan receptor protein non-expression cell or its cell membrane fractions, such test compound is selected as a test compound having agonist activity. On the other hand, in the case of setting cAMP production promoting function as an index, when the amount of produced cAMP is not less than 105% in the case of adding a test compound (where the amount of cAMP without adding a test compound is set as 100% as an index), and when cell-stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a test compound having agonist activity.

In the case of using cell-stimulating activity assay system characterized by introducing the CRE-reporter gene described in above (D)-(4), (when the cAMP production inhibition function is set as an index and where the

luminescence occurred by the forskolin reaction is set as 100%), when the amount of luminescence is no more than 95% and when a stimulating activity is not observed in the case of using an orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a test compound having agonist activity. On the other hand, (when the cAMP production promoting function is set as an index and where the amount of luminescence is set as 100% when a test compound is not added), when the amount of luminescence is not less than 105% and when a stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such compound is selected as the test compound having agonist activity.

In the case of using cell-stimulating activity assay system characterized by measuring the arachidonic acid release described in above (D)-(5), (where the amount of [H^3] arachidonic acid metabolite in a medium by a test compound non-adding reaction buffer is set as 100%), when the amount of [H^3] arachidonic acid metabolite in the medium in which a test compound is added is not less than 105% and when cell-stimulating activity is not observed in the case of using an orphan receptor protein non-expression cell or its cell membrane fractions, such test compound is selected as a test compound having agonist activity.

In the case of using cell-stimulating activity assay system characterized by measuring the Ca^{2+} release described in above (D)-(6), (where the intensity of luminescence without adding the test compound is set as 100%), when the intensity of luminescence of the compound which the test compound added is not less than 105% and when cell-stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a test compound having agonist activity.

In the case of using cell-stimulating activity assay system characterized by measuring the production of inositol phosphate described in above (D)-(7), (where a radioactivity in a medium by a test compound non-adding reaction buffer is set as 100%), when a radioactivity in a medium which a test compound is added is not less than 105% and when cell-stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fraction, such test compound is selected as a test compound having agonist activity.

[0027]

(F) Structural comparison of a test compound having an agonist function:

After selecting test compounds having agonist activity as described in the above (E), by comparing the structures of the test compounds having the agonist activity, a common structure of the test compounds is determined to obtain or prepare a ligand candidate compound of said common structure.

Said ligand candidate compound refers to a compound having the common structure to test compounds having agonist activity and having stronger cell-stimulating activity according to the above (D) as compared with other test compound (e.g. natural peptide, natural protein and natural non-peptide compound).

In the case where test compounds having agonist activity are natural/non-natural peptide or natural/non-natural protein, the amino acid sequences encoding the peptides or proteins are compared, and then a partial sequence which is highly homologous or a part having a similar steric structure is considered to be the common structure.

More specifically, considering a common structure when the orphan receptor protein is GHS receptor (Howard, A. D. et al. Science 273: 974-977, 1996), for example, the common structure that "having RF amide structure at C-terminus"

can be deduced by comparing the SEQ ID NO: 1, 6, 9-10, 13, 16-20. From said common structure, according to the method of screening or the method of determination of a ligand of the present invention, when the orphan receptor protein is the GHS receptor, it is considered to be the ligand candidate compound containing a common structure, moreover, the ligand of GHS receptor (endogenous) is the peptide containing the RF-amide structure at C-terminus.

As the peptide containing the RF-amide structure at the C-terminus, there are A-18-F-NH_2, F-8-F-NH_2, (Perry, S. J. et al. FEBS Lett. 409: 426-430, 1997), prolactin-releasing peptide (Hinuma, S, et al. Nature 393: 272-276, 1998) are known for mammal. For lower animals, there are groups of RF amide-peptide family ubiquitously; so that it is possible that there might be GHS receptor (endogenous) ligands within novel RF-amide peptides.

If the test compounds having agonist activity are a natural/non-natural non-peptide compound or a synthetic compound, the chemical structures of those compounds are compared, to identify a basic structure (e.g. specific cyclic structures, for example, cycloalkyl as "alicyclic hydrocarbon", cycloalkenyl, cycloalkandienyl and the like as saturated or unsaturated alicylic hydrocarbon, aromatic heterocycle as "heterocycle", saturated or unsaturated non-aromatic heterocycle (aliphatic heterocycle) etc.) as a common structure.

Also, since the identification of a ligand of the present invention is made using the above common structure as an index, it is much easier and much more certain to obtain a subtype of ligand having structural similarity with the ligand as compared with a current method.

[0028]

(G) Processes after determining the common structure of the test compound having agonist activity:

A method of preparing or obtaining a ligand candidate compound having a common structure determined according to the above (F) is specifically described as follows.

(1) A method of preparing or obtaining a ligand candidate compound by selecting a ligand candidate compound having a common structure by searching a natural peptide, a natural protein and a natural non-peptide compound having a common structure

A ligand and its subtype can be estimated by searching a natural peptide, a natural protein and a natural non-peptide compound having a common structure based on a common structure of test compounds described in above (F).

There are quite a few publicly know databases which can be used. The representative databases are, for example, Beilstein Handbook of Organic Chemistry (Beilstein), CROPR (Serwent Crop Protection Registry) file (Derwent), Derwent Drug file (Derwent) and the Merck Index (Merck).

Then, for those estimated ligand, it is possible to determine whether or not it is a ligand or its subtype by measuring its cell-stimulating activity, and comparing it with cell-stimulating activity of a ligand candidate compound using the cell-stimulating activity assay system described in above (D).

[0029]

(2) A method of obtaining a ligand candidate compound by cloning cDNA or a gene encoding a ligand or its subtype by preparing a primer or a probe having a base sequence encoding a common structure (where the test compound having agonist activity is a peptide or a protein).

First, a primer or probe is prepared, which has a common structure of test compounds having agonist activity described in above (F), that is, a base sequence encoding a partial sequence which is highly homologous to the amino acid sequence encoding the test compounds having agonist activity.

Then using the primer or probe, amplification is performed by known PCR techniques for a DNA encoding a ligand candidate compound derived from any tissues (e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract, blood vessel, heart, etc.) of human, other warm-blooded animals (e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.) and fish, or a genomic DNA library, cDNA library derived from cells and the like.

The cDNA comprising the base sequence encoding a cloned ligand candidate compound can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may contain ATG as a translation initiation codon at the 5' end thereof and TAA, TGA or TAG as a translation termination codon at the 3' end thereof. These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

According to the preparation method for a cells capable of expressing an orphan receptor protein described in above (A) and (B), it is possible to cultivate a transformant containing the DNA encoding a ligand candidate compound, and separate and purify the ligand candidate compound from the cultivated product.

That is, after cultivation, the transformants or the cells are collected by a publicly known method, and suspended in a appropriate buffer, and then disrupted by publicly known methods such as (ultra)sonication, a treatment with lysozyme and/or freeze-thaw cycling, followed by centrifugation, filtration, etc. Thus, the crude extract of the estimated ligand or its subtypes can be obtained. The buffer used for the procedures may contain a protein modifier such as urea or guanidine hydrochloride, or a surfactant such as Triton X-100™, etc.

The supernatant or the ligand candidate compound contained in the extract thus obtained can be purified by appropriately combining the publicly known methods for separation and purification. Such publicly known methods for separation and purification include a method utilizing difference in solubility such as salting out, solvent precipitation, etc.; a method mainly utilizing difference in molecular weight such as dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, etc.; a method utilizing difference in electric charge such as ion exchange chromatography, etc.; a method utilizing difference in specific affinity such as affinity chromatography, etc.; a method utilizing difference in hydrophobicity such as reverse phase high performance liquid chromatography, etc.; a method utilizing difference in isoelectric point such as isoelectrofocusing electrophoresis; and the like.

When the ligand candidate compound thus obtained is in a free form, it can be converted into a salt by publicly known methods or modifications thereof. On the other hand, when the polypeptide is obtained in the form of a salt, it can be converted into a free form or in the form of a different salt by publicly known methods or modifications thereof.

The ligand candidate compound can be prepared by publicly known methods for protein synthesis, or by cleaving the protein containing the ligand candidate compound with an appropriate peptidase. For the methods for protein synthesis, for example, either solid phase synthesis or liquid phase synthesis may be used. That is, the partial peptide or amino acids that can construct the ligand candidate compound are condensed with the remaining part of the partial peptide of the present invention. Where the product contains protecting groups, these protecting groups are removed to give the desired ligand candidate compound. Publicly known methods for condensation and

elimination of the protecting groups are described in 1) - 5) below.

1) M. Bodanszky & M.A. Ondetti: Peptide Synthesis, Interscience Publishers, New York (1966)

2) Schroeder & Luebke: The Peptide, Academic Press, New York (1965)

3) Nobuo Izumiya, et al.: *Peptide Gosei-no-Kiso to Jikken* (Basics and experiments of peptide synthesis), published by Maruzen Co. (1975)

4) Haruaki Yajima & Shunpei Sakakibara: *Seikagaku Jikken Koza* (Biochemical Experiment) 1, *Tanpakushitsu no Kagaku* (Chemistry of Proteins) IV, 205 (1977)

5) Haruaki Yajima ed.: *Zoku Iyakuhin no Kaihatsu* (A sequel to Development of Pharmaceuticals), Vol. 14, Peptide Synthesis, published by Hirokawa Shoten

After completion of the reaction, the product may be purified and isolated by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography and recrystallization to give the ligand candidate compound. When the ligand candidate compound obtained by the above methods is in a free form, the peptide can be converted into an appropriate salt by a publicly known method; when the protein is obtained in a salt form, it can be converted into a free form or a different salt form by a publicly known method.

Using the assay system of cell-stimulating activity described in above (D) for a ligand candidate compound, it is possible to find out whether the ligand candidate compound has an agonist (ligand) activity by measuring cell-stimulating activity and comparing it with that of a test compound. It is possible to find out whether a ligand candidate compound the agonist (ligand) activity of which is observed, is a (endogenous) ligand, its subtype for an

orphan receptor protein or not, by the ligand identification method described later in (J).

Moreover, mRNA of the desired gene is purified using the probes such as Gene Trapper to acquire cDNA from the mRNA purified. Further, according to the preparation method for the orphan receptor protein described in above (A), it is possible to obtain a ligand candidate compound by culturing the transformant, which contain the DNA encoding the ligand candidate compound.

[0030]

(3) A method of searching a ligand candidate compound by searching sequence database for a peptide or a protein having a common structure (when a test compound having the agonist activity is a peptide, a protein or their salt)

A ligand candidate compound can be identified by searching for a peptide or a protein containing a common structure of a test compound having agonist activity described in above (F) (i.e., a protein or a peptide containing the base sequence encoding the partial sequence which is highly homologue to the amino acid sequence encoding a test compound having agonist activity) in database.

Sequence databases are, for example, GenBank (registered trade mark) file (National Institute of Health), VTS (virtual transcribed sequence).

When the base sequence of a ligand candidate compound is once identified, it is possible to obtain a ligand candidate compound according to the method described in above (G)-2.

Moreover, as a result of searching database, when the sequence which is supposed to encode a part of ligand candidate compound, is discovered, it is possible to obtain a ligand candidate compound by preparing a primer or a probe based on said sequence and in accordance with the method described in above (G)-2,.

[0031]

(5) A method of searching a ligand candidate compound by preparing an antibody which recognizes a common structure

A common structure of a test compound having agonist activity described in above (F) (i.e., a peptide represented by a partial sequence which is homologues to the amino acid sequence encoding a test compound having agonist activity) is prepared according to the synthesis methods for preparing peptides (proteins) as described above.

Then, an antibody to said peptide is prepared according to the following method. Antibodies to the peptide may be any of polyclonal antibodies and monoclonal antibodies, as long as they are capable of recognizing the peptide. The antibodies to the peptide may be prepared by publicly known methods for preparing antibodies or antisera, using as antigens the peptide.

[Preparation of monoclonal antibody]

(a) Preparation of monoclonal antibody-producing cells

The peptide is administered to mammals either solely or together with carriers or diluents to the site where the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once in every two to six weeks and 2 to 10 times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats and fowls, with mice and rats being preferred.

In the preparation of monoclonal antibody-producing cells, warm-blooded animals, e.g., mice, immunized with an antigen wherein the antibody titer is noted is selected, then the spleen or lymph node is collected after 2 to 5 days from the final immunization and antibody-producing cells contained therein are fused with myeloma cells to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may be made, for example,

by reacting a labeled form of the peptide, which will be described later, with the antiserum followed by assaying the binding activity of the labeling agent bound to the antibody. The fusion may be operated, for example, by the known Koehler and Milstein method (Nature, 256, 495, 1975). Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc., of which PEG is preferably employed.

[0032]

Examples of the myeloma cells include myeloma cells of warm-blooded animals such as NS-1, P3U1, SP2/0, AP-1, etc. In particular, P3U1 is preferably employed. A preferred ratio of the count of the antibody-producing cells used (spleen cells) to the count of myeloma cells is within a range of approximately 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of approximately 10 to 80% followed by incubating at about 20 to about 40℃, preferably at about 30 to about 37℃ for about 1 to about 10 minutes, an efficient cell fusion can be carried out.

Various methods can be used for screening of a monoclonal antibody-producing hybridoma. Examples of such methods include a method which comprises adding the supernatant of hybridoma to a solid phase (e.g., microplate) adsorbed with the peptide as an antigen directly or together with a carrier, adding an anti-immunoglobulin antibody (when mouse cells are used for the cell fusion, anti-mouse immunoglobulin antibody is used) labeled with a radioactive substance or an enzyme, or Protein A and detecting the monoclonal antibody bound to the solid phase, and a method which comprises adding the supernatant of hybridoma to a solid phase adsorbed with an anti-immunoglobulin antibody or Protein A, adding the receptor protein labeled with a radioactive substance or an enzyme and detecting the monoclonal antibody bound to the solid phase.

The monoclonal antibody can be selected by publicly known methods or by modifications of these methods. In general, the selection can be effected in a medium for animal cells supplemented with HAT (hypoxanthine, aminopterin and thymidine). Any selection and growth medium can be employed as far as the hybridoma can grow therein. For example, RPMI 1640 medium containing 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium (Wako Pure Chemical Industries, Ltd.) containing 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku Co., Ltd.) and the like can be used for the selection and growth medium. The cultivation is carried out generally at 20°C to 40°C, preferably at about 37°C, for 5 days to 3 weeks, preferably 1 to 2 weeks. The cultivation can be conducted normally in 5% CO_2. The antibody titer of the culture supernatant of hybridomas can be determined as in the assay for the antibody titer in antisera described above.

[0033]

(b) Purification of monoclonal antibody

Separation and purification of a monoclonal antibody can be carried out by methods applied to conventional separation and purification of immunoglobulins [e.g., salting-out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (e.g., DEAE), ultracentrifugation, gel filtration, or a specific purification method which comprises collecting only an antibody with an activated adsorbent such as an antigen-binding solid phase, Protein A, Protein G, etc. and dissociating the binding to obtain the antibody].

[0034]

[Preparation of polyclonal antibody]

The polyclonal antibody of the peptide above can be manufactured by publicly known methods or modifications thereof. For example, a complex of immunogen (peptide

antigen) and a carrier peptide is prepared, and a mammal is immunized with the complex in a manner similar to the method described above for the manufacture of monoclonal antibodies. The product containing the antibody to the receptor protein of the present invention is collected from the immunized animal followed by separation and purification of the antibody.

In the complex of an immunogen and a carrier protein used to immunize a mammal, the type of carrier peptide and the mixing ratio of a carrier to hapten may be any type and in any ratio, as long as the antibody is efficiently produced to the hapten immunized by crosslinking to the carrier. For example, bovine serum albumin, bovine thyroglobulins, keyhole limpet hemocyanin, etc. is coupled to hapten in a carrier-to-hapten weight ratio of approximately 0.1 to 20, preferably about 1 to about 5.

A variety of condensing agents can be used for the coupling of a carrier to hapten. Glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group, etc. are used for the coupling.

The condensation product is administered to warm-blooded animals either solely or together with carriers or diluents to the site in which the antibody can be produce by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvant or incomplete Freund's adjuvant may be administered. The administration is usually made once approximately in every 2 to 6 weeks and about 3 to about 10 times in total.

The polyclonal antibody can be collected from the blood, ascites, etc., preferably from the blood of mammals immunized by the method described above.

The polyclonal antibody titer in antiserum can be assayed by the same procedure as that for the determination of serum antibody titer described above. The separation

and purification of the polyclonal antibody can be carried out, following the method for the separation and purification of immunoglobulins performed as applied to the separation and purification of monoclonal antibodies described hereinabove.

Thus, using the cross-linking reaction for the antigen recognizing the structure of the obtained compound having agonist activity, a ligand candidate compound can be detected. And using the varieties of extraction methods and chromatography, the ligand candidate can be obtained using the combination of the varieties of extraction methods and chromatography.

[0035]

(H) Screening method for a compound which promotes or inhibits a function of a orphan receptor protein:

It is possible to conduct a screening of a compound which promotes a function of an orphan receptor protein (highly active agonist) or a compound (agonist) which inhibits a function of an orphan receptor using the ligand candidate compound obtained in above (G).

A compound which promotes a function of an orphan receptor protein is described as "highly active agonist". The term "highly active" means that it shows a stronger cell-stimulating activity (moreover, cell-stimulating activity described in above (D)) as comparing to "the test compound having agonist activity" described in above (E).

The screening method is described bellow.

It is possible to effectively conduct screening of a compound which alters binding level between the ligand candidate compound obtained in above (G) and an orphan receptor protein (e.g. peptide, protein, non-peptide compound, synthetic compound, fermentation product, etc.) or its salts, using an orphan receptor protein or a receptor binding assay using an expression system constructed using a recombinant orphan receptor protein expression system.

That is, cell stimulating activities as described in above (D) such as (a) changes in extracellular pH, (b) arachidonic acid release, (c) acetylcholine release (d) intracellular Ca^{2+} release, (e) changes in intracellular cAMP (level), (f) changes in intracellular cGMP (level), (g)inositol phosphate production, (h)changes in cell membrane potential, (i) phosphorylation of intracellular proteins, (j) activation of c-fos, (k) GTPγS bonds (l) expression of reporter gene as an index, are measured when an orphan receptor protein expression cells or its cell membrane fractions is brought in contact with a ligand candidate compound and when said orphan receptor protein expression cells or its cell membrane fractions is brought in contact with a compound of a candidate compound which promotes and inhibits the function of said orphan receptor protein.

If cell-stimulating activity when a compound of a candidate compound which accelerates and inhibits the function of said orphan receptor protein is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions as compared to cell-stimulating activity when a ligand candidate compound is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, the candidate compound is possible to be a compound which promotes the function of said orphan receptor protein (what is called agonist).

To find out whether said candidate compound is a compound which (selectively and specifically) promotes the function of said orphan receptor protein (what is called agonist) or not, the amount of specific binding between the said orphan receptor protein and said candidate compound is determined.

As the method for determining the amount of specific binding, for example, there is a method of determining the amount of specific binding when a labeled candidate

compound is brought in contact with an orphan receptor protein. As a result, if there is a sufficient binding amount (over 1% increase, preferably 10% increase in the binding amount against non-specific binding), said candidate compound is recognized as a compound which promotes the function of the orphan receptor protein (what is called agonist).

The detail of said method for determination is as follows.

First, an orphan protein receptor being used for the determination can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed a large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above. It is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA fragment encoding the orphan receptor protein into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SRα promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, the subject containing an orphan receptor protein may be an orphan receptor protein purified by

publicly known method, cells containing a orphan receptor protein or membrane fractions of such cells.

The cells containing an orphan receptor protein are host cells which are expressing the orphan receptor protein. Preferred are insect cells and animal cells.

The cell membrane fractions are a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the orphan receptor protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell.

As the labeled candidate compound, for example, the candidate compound, which is labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc., is used.

To conduct the method for determination, first, a standard receptor preparation is prepared by suspending cells containing the orphan receptor protein or the membrane fraction thereof in a buffer appropriate for use

in the determination method. Any buffer can be used so long as it does not interfere with candidate -receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80™ (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. A given amount (5,000 to 500,000 cpm) of the labeled candidate compound is added to 0.01 ml to 10 ml of the receptor solution. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity in the glass fiber filter paper is then measured by means of a liquid scintillation counter or γ-counter.

On the other hand, when a ligand candidate is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions and show a weak or no cell simulating activity compared with cell-stimulating activity measured when a ligand candidate compound is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, the candidate compound could be a compound which inhibits the function of the orphan receptor protein (what is called, antagonist).

Moreover, it is possible that candidate compound is the compound which inhibits the function of the orphan receptor protein (what is called, antagonist), where a week or no cell-stimulating activity is observed when cells

capable of expressing an orphan receptor or its cell membrane fractions are brought in contact with a compound of candidate compound which promotes or inhibits the function of said orphan receptor protein or a ligand candidate compound.

To find out whether said candidate compound is a compound which (selectively and specifically) inhibits the function of said orphan receptor protein (what is called antagonist) or not, the amount of specific binding between the said orphan receptor protein and said candidate compound is measured.

As the method for determining the amount of specific binding is, for example, there is a method of measuring the amount of specific binding between a labeled candidate compound and an orphan receptor protein. As a result, if there is a sufficient binding amount (over 1% increase, preferably 10% increase in the binding amount against non-specific binding), said candidate compound is recognized as a compound which promotes the function of orphan receptor protein (what is called antagonist).

The same method as the method for conducting screening of a compound which promotes the function of orphan receptor protein (what is called, high activation agonist) described above is used.

Moreover, it is possible to conduct screening of a compound which inhibits the function of an orphan receptor protein by measuring and comparing the amount of binding between a labeled ligand candidate compound and said orphan receptor protein when a labeled ligand is brought in contact with an orphan receptor protein and when the labeled ligand candidate compound and the candidate compound of a compound which inhibits the function of an orphan receptor protein are brought in contact with an orphan receptor protein.

The detail of said screening method is described bellow.

First, an orphan protein receptor being used for the determination can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed in a large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above. It is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA fragment encoding the orphan receptor protein into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SRα promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, in the above screening method, the subject containing the orphan receptor protein may be the orphan receptor protein purified by publicly known method, cells containing the orphan receptor protein or membrane fraction of such cells.

The cells containing the orphan receptor protein are host cells that have expressed the orphan receptor protein. Preferred are insect cells, animal cells and the like.

The cell membrane fractions are a fraction abundant in cell membrane obtained by a cell disruption and a subsequent fractionation by a publicly known method.

Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is affected mainly by using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in an orphan receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing an orphan receptor protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell. As the amount of expression increases, a ligand binding activity per unit of membrane fraction (specific activity) increases so that not only a highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot.

To perform screening of a compound which alters a binding property between a ligand candidate compound and an orphan receptor, an appropriate orphan receptor fraction and a labeled ligand or its subtype is required.

As a labeled ligand candidate compound, the compound labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc, is used.

More specifically, to conduct a screening of a compound which inhibits the function of an orphan receptor protein, first, cells containing an orphan receptor proteins and its

cell membrane fractions are suspended with a buffer appropriate for a screening to prepare a standard receptor protein. Any buffer can be used so long as it does not interfere with ligand-receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80™ (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. To 0.01ml - 10ml of the receptor solution, a given amount (5,000 to 500,000 cpm) of labeled ligand is added, and 10^{-4} M - 10^{-10} M of a candidate compound which inhibits a function of an orphan receptor is simultaneously added to be co-present. To examine non-specific binding (NSB), a reaction tube containing an unlabeled test compound in a large excess is also prepared. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to about 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity on the glass fiber filter paper is then measured by means of a liquid scintillation counter or γ-counter. Regarding the count obtained by subtracting the amount of non-specific binding (NSB) from the count obtained in the absence of any competitive substance (B_0) as 100%, when the amount of specific binding (B-NSB) is, for example, 50% or less, the candidate compound for the compound inhibit the function of orphan receptor protein can be selected as a the compound inhibit the function of orphan receptor protein (what is called, antagonist).

[0036]

(I) The test the candidate compound for the compound which inhibits the function of orphan receptor protein described in above (H), the compound which promotes or inhibits an orphan receptor protein:

As the candidate substance for the compound which inhibits the function of orphan receptor protein described in above (H), is selected from natural/non-natural peptides, natural/non-natural proteins, natural/non-natural non-peptide compounds, synthetic compounds and natural/non-natural fermentation products.

The compound which promotes or inhibits function of an orphan receptor protein means a compound which is recognized as a compound which promotes a function of an orphan receptor protein and a compound which inhibits a function of an orphan receptor protein according to the screening method described in above (H). The compound can form salts thereof.

Examples of the salts of the compound are physiologically acceptable bases (e.g., alkali metals) and acids (e.g., organic acids and inorganic acids). Of these salts, preferred are physiologically acceptable acid addition salts. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid and sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

Moreover, a compound which promotes a function of an orphan receptor protein (highly active agonist) and a compound which inhibits a function of an orphan receptor protein (antagonist) obtainable by the method of above (H) have a function similar to a physiological activity of a (endogenous) ligand or its subtype later described. Thus,

it is useful as a safe and low toxic pharmaceutical preparation according to the ligand activity.

An antagonist against an orphan receptor protein can suppress the physiological activity of the ligand or its subtype against the orphan receptor protein. Thus, it is useful as a safe and low toxic pharmaceutical preparation which suppress the ligand activity.

A highly active agonist against an orphan receptor protein is useful as a safe and low toxic pharmaceutical preparation for increasing the physiological activity of the ligand of the orphan receptor protein.

[0037]

In the case of using the antagonist and the highly active agonist obtainable according to the method of the present invention as a pharmaceutical composition, it is administered according to a conventional procedure. For example, the compound or its salts may be prepared into tablets, capsules, elixirs, microcapsules, sterile solutions, suspensions, etc.

Since the preparation thus obtained is safe and low toxic, it can be administered to human or warm-blooded animal (e.g., rat, mouse, rabbit, sheep, swine, bovine, cat, dog, monkey, etc.).

The amount of administration of the ligand or its subtype, the antagonist and the agonist may vary depending on a subject to be administered, target organ, target disease, route of administration and the like. For example, in oral administration, the dose is normally about 0.1 mg to about 100 mg, preferably about 1.0 to about 50 mg, and more preferably about 1.0 to about 20 mg per day. For non-oral administration, the single dose may vary depending on a subject to be administered, target organ, target disease, route of administration and the like. In parenteral administration, it may be intravenously administered at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, and more preferably

about 0.1 to about 10 mg. For other animal species, the corresponding dose as converted per 60 kg body weight can be administered.

[0038]

(J) The method of determination of the ligand of orphan receptor protein or its subtype:

The present invention provides not only the screening method for a compound which promotes or inhibits a function of an orphan receptor protein as described in above (H), but also, using as index a common structure of test compounds having agonist activity, a method of determining a ligand of the orphan receptor protein or its subtype effectively and securely.

That is, the present invention provides a method of identifying a ligand of an orphan receptor or its subtypes by

(i) contacting a test compound with cells capable of expressing an orphan receptor or its cell membrane, to measure cell-stimulating activity mediated by said orphan receptor,

(ii) comparing the cell stimulating activities measured for each test compound, to determine a common structure of the test compounds having agonist activity, and

(iii) measuring amount of specific binding between a ligand candidate compound having the common structure thus identified and the orphan receptor protein.

In the identification method, the same method as described above may be used with respect to the steps: (i) contacting a test compound with cells capable of expressing an orphan receptor or its cell membrane, to measure cell-stimulating activity mediated by said orphan receptor, and

(ii) comparing the cell stimulating activities measured for each test compound, to identify common structure of the test compounds having agonist activity.

Moreover, the method to determine whether said ligand candidate compound is a (endogenous) ligand or its subtype using the ligand candidate compound comprising a common structure (above), is as follows.

Whether the above ligand candidate compound is a specific ligand for an orphan receptor protein or not, it is possible to determine by measuring amount of specific binding for the ligand candidate compound to said orphan receptor protein.

That is, the method includes a method of determine the binding amount for a labeled ligand candidate compound to said orphan receptor protein when a labeled ligand candidate compound is brought in contact with the orphan receptor protein. As a result of determination, it is recognized as a ligand if there is sufficient amount of binding (over 1% increase, preferably 10% increase in the binding amount against non-specific binding).

On the other hand, when enough binding amount is not observed, said ligand candidate compound may possibly be a substance having cell-stimulating activity and a low binding amount to said orphan receptor protein, that is, a non-specific agonist-like substance.

The identification method will be described in more detail bellow.

First, an orphan protein receptor used for the said identification method can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed in large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above, it is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be

used. For introducing a DNA fragment encoding the orphan receptor protein into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SRα promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, the subject containing the orphan receptor protein may be the orphan receptor protein purified by publicly known method, cells containing the orphan receptor protein or membrane fraction of such cells.

The cells containing the orphan receptor protein mean host cells that have expressed the orphan receptor protein. Preferred are insect cells, animal cells and the like.

The cell membrane fractions are a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30

minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the orphan receptor protein and in the membrane fraction is preferably 10^3 to 10^8 molecules per cell, more preferably 10^5 to 10^7 molecules per cell.

As the labeled ligand candidate compound, labeled ligand candidate compound which is labeled with $[^3H]$, $[^{125}I]$, $[^{14}C]$, $[^{35}S]$, etc., is used.

To conduct the measurement method , first, a standard receptor preparation is prepared by suspending cells containing the orphan receptor protein or the membrane fraction thereof in a buffer appropriate for screening. Any buffer can be used so long as it does not interfere with candidate-receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80™ (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. A given amount (5,000 to 500,000 cpm) of the labeled candidate compound is added to 0.01 ml to 10 ml of the receptor solution. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity in the glass fiber filter paper is then

measured by means of a liquid scintillation counter or γ -counter.

[0039]

In the specification and drawings, the codes of bases and amino acids are denoted in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature or by a common codes in the art, examples of which are shown below. For amino acids that may have optical isomers, L form is presented unless otherwise indicated.

DNA : deoxyribonucleic acid
cDNA : complementary deoxyribonucleic acid
A : adenine
T : thymine
G : guanine
C : cytosine
RNA : ribonucleic acid
mRNA : messenger ribonucleic acid
dATP : deoxyadenosine triphosphate
dTTP : deoxythymidine triphosphate
dGTP : deoxyguanosine triphosphate
dCTP : deoxycytidine triphosphate
ATP : adenosine triphosphate
EDTA : ethylenediaminetetraacetic acid
SDS : sodium dodecyl sulfate

[0040]

Gly : glycine
Ala : alanine
Val : valine
Leu : leucine
Ile : isoleucine
Ser : serine
Thr : threonine
Cys : cysteine
Met : methionine
Glu : glutamic acid
Asp : aspartic acid

Lys : lysine
Arg : arginine
His : histidine
Phe : phenylalanine
Tyr : tyrosine
Trp : tryptophan
Pro : proline
Asn : asparagine
Gln : glutamine
pGlu : pyroglutamic acid
HEPES: N-[2-hydroxyethyl] piperazine-n'-[2-ethanesulfonic acid]
HBSS: Hank's Balanced Salt Solution

[0041]

The sequence identification numbers in the sequence listing of the specification indicate the following sequences, respectively.

[SEQ ID NO:1]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:2]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:3]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:4]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:5]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:6]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:7]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:8]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:9]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:10]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:11]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:12]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:13]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:14]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:15]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:16]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:17]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:18]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:19]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:20]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:21]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:22]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:23]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:24]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:25]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:26]

This shows the amino acid sequence of the sample used in Example 2, later described (see Table 1 and Table 2).

[SEQ ID NO:27]

This shows the base sequence of DNA encoding GHCF used in Example 1, later described.

[SEQ ID NO:28]

This shows the base sequence of DNA encoding GHR used in Example 1, later described.

[0042]

[EXAMPLES]

The present invention will be described in detail below with reference to EXAMPLES, but is not deemed to limit the scope of the present invention thereto.

EXAMPLE 1 Preparation of GHS receptor expression CHO cells

Rat GHS receptor (growth hormone secretarogue receptor) was obtained as follows. Following two kinds of synthetic DNA were synthesized according to the base sequence of rat GHS receptor type 1A reported in Molecular Endocrinology. 11 (4), 415-423 (1997).

GHCF:5'-GTCGACCATGTGGAACGCGACCCCCAGCGAGGAG-3' (SEQ ID NO:27)

GHR:5'-GCTAGCGGAGAGATGGGATGTGCTGTCATGT-3' (SEQ ID NO:28)

Using these synthetic DNAs, it was obtained from rat hypothalamus cDNA with PCR.

The reaction solution was prepared by mixing 2 μl of cDNA solution for rat hypothalamus (0.2 ng poly $(A)^+$ RNA derived), 1 μl GHCF (10 μM), 1 μl GHR (10 μM), 5 μl of an attached 10x reaction solution, 5 μl of dNTP (10mM), 1 μl of Klen Taq (Clonetech) and 35 μl of distilled water, to make a total volume of 50 μl.

The PCR was carried out with ThermalCycler9600. The temperature was set at 95℃ for 2 minutes for denaturation, then the cycle set (98°C for 10 seconds followed by 68°C for 90 seconds) was repeated 35 times. After checking the amplification of PCR product of 1.1 kb (sized) in an aliquot of the PCR product by electrophoresing, the PCR product was subcloned to Escherichia coli using TA cloning kit (Invitrogen Inc.). From Escherichia coli obtained from subcloning, the plasmid was extracted using plasmid extracting machine (Kurabo Co., Ltd.) and the base sequence of the insertion fragment was determined. The sequence was matched with GHS receptor cDNA reported in the above literature. After cleaving by Sal I and Nhe I, the fragment of 1.1kb GHS receptor cDNA fragment was obtained. Moreover, the multi-cloning site of pAKKO-111H, the expression vector for animal cells, was cleaved at the restriction enzyme sites Sal I and Nhe I and electophoresed to recover the vector portion. The rat GHS receptor cDNA fragment and the

expression vector were ligated. Then, Escherichia coli JM109 was transformed to obtain E.coliJM 109-pAKKOGHSR.

Transformant E.coliJM 109-pAKKOGHSR was cultured to prepare a large amount of plasmid DNA of pAKKOGHSR.

20 μg of the plasmid DNA prepared was dissolved in 1ml of phosphate buffered saline (PBS), transferred into the vial of gene transfer (Wako Pure Chemical Industries, Ltd.), and shaken vigorously by a vortex mixer to form a lyposome containing DNA. CHO/dhfr$^-$ cells of 1 or 2 x 10^6 were inoculated on cell culture dish having a diameter of 35 mm. After 20 hours of cultivation, the medium was exchanged to the new medium. The lyposome solution (25 μl) having 0.5μg DNA was added to each of the dishes and incubated for 16 hours to introduce plasmid DNA into the cell. Furthermore, the medium was exchanged and cultured for one day in the new medium. The medium was exchanged to a selection medium (minimum essential medium without deoxyribibonucleosides and ribonucleosides, alpha medium with 10% dialyzed fetal bovine serum) and said cells were cultured for 3 days. Finally, said cells were treated with trypsin for digestion. The cells were inoculated on the selection medium at low density to select the transformant. Only the transformants were able to survive in the medium. Thus, by subcloning repeatedly, CHO-GHSR cells were established.

EXAMPLE 2

GHS receptor expressing CHO cells were inoculated in the capsules for a cytosensor (2.7 x 10^5 cells/capsule), before incubating them overnight. After mounting the cells on the cytosensor, a low buffered RMPI 1640 medium supplemented with 0.1% bovine serum albumin was set in the flow path of the cytosensor, and supplied to the cells in a pump cycle of ON (80 seconds) and OFF (40 seconds) to measure acidification rates. The rate of change in extracellular pH (from 8 to 30 seconds after the pump

stopped) was calculated as an acidification rate. The change in the acidification rate was monitored, while the flow path was switched to have the sample expose to the cells for 7 minutes and 2 seconds. In order to compare the acidification rate of the each well, the data for 3 cycles immediately before injection of the sample was standardized as 100%. After the values of the acidification rates have been stabilized, a high concentration (0.1 mM - 1 μM) of peptide sample was exposed to the cells, and the reaction was measured using the cytosensor (see Table 1 and Table 2). The reaction was also measured for CHOdhfr$^-$ cells which do not express GHS receptors using the cytosensor according to the same method. (○) indicates the cases in which the acidification rate (at a peak while the reaction took place) was more than 120 %. (△) indicates the cases in which the rate was not more than 120%, however, there was noticeable reaction. (×) indicates the cases in which there was no reaction. (?) indicates the cases in which it was hard to determine one of the above categories. Note that HwAWfKa in Table 1 and Table 2 indicates His D-Trp Ala Trp D-Phe Lys-amide.

[0044]

[Table 1]

[Table 2]

[0045]

[EFFECTS OF THE INVENTIOON]

According to the present invention, a ligand or its subtype of an orphan receptor protein, antagonist and highly active agonist can be obtained effectively and securely, by contacting test compounds with cells expressing an orphan receptor protein, its cell membrane fractions or orphan receptor proteins expressed on cells expressing the orphan receptor protein or its cell membrane fractions, measuring orphan receptor protein-mediated

cell-stimulating activities of the test compounds, comparing the cell stimulating activities measured for each test compound to identify agonists, and then comparing the structures of the agonists.

[0046]

[SEQUENCE LISTING]

[Document Name] ABSTRACT
[Abstract]
[Problem]

The present invention provides a method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein.

[Means]

The screening method of the present invention is, for example, the following: A method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein, comprising:

(i) measuring cell-stimulating activity when test compound (a) is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, and when test compound (a) is brought in contact with cells which are not capable of expressing the orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound (a), to identify compounds having agonist activity, and

(iii) ① comparing cell-stimulating activity when a ligand candidate compound which is selected by considering a common structure of said compound(s) having agonist activity is brought in contact with said cells capable of expressing the orphan receptor or its cell membrane fractions, and cell-stimulating activity when test compound (b) is brought in contact with said cells capable of expressing the orphan receptor or its cell membrane fractions, and
② measuring the amount of specific binding between said orphan receptor protein and the test compound (b).

[Effect]

That is, according to the present invention, a ligand or its subtype of an orphan receptor protein, antagonist and highly active agonist can be obtained effectively and securely, by contacting test compounds with cells

expressing an orphan receptor protein, its cell membrane fractions or orphan receptor proteins expressed on cells expressing the orphan receptor protein or its cell membrane fractions, measuring orphan receptor protein-mediated cell-stimulating activities of the test compounds, comparing the cell stimulating activities measured for each test compound to identify agonists, and then comparing the structures of the agonists.

[Selected Figure]

None.

JP 1999-236597

Table 1

Structure	Name	CHSR	CHO dhfr⁻
HwAWfKa (Bachem)	GHRP-6	○	×
FMRFa (SEQ ID No: 1) (Bachem)		○	×
YFMRFa (SEQ ID No: 2) (Bachem)		○	?
YGGFMRFa (SEQ ID No: 3) (Bachem)		○	?
YGGFMRF (SEQ ID No: 4) (Bachem)		×	×
PQRFa (SEQ ID No: 5) (Bachem)		×	×
FLFQPQRFa (SEQ ID No: 6) (Bachem)	F-8-F-NH_2	○	×
AGEGLSSPFWSLAAPQRFa (SEQ ID No: 7) (Bachem)	A-18-F-NH_2	△	○
pEDPFLRFa (SEQ ID No: 8) (Bachem)		×	×
DRNFLRFa (SEQ ID No: 9) (Bachem)		○	×
NRNFLRFa (SEQ ID No: 10) (Bachem)		○	×
TNRNFLRFa (SEQ ID No: 11) (Bachem)		○	?
PDVDHVFLRFa (SEQ ID No: 12) (Bachem)		×	×
KNEFIRFa (SEQ ID No: 13) (Bachem)	AF-1	○	×
KHEYLRFa (SEQ ID No: 14) (Bachem)	AF-2	?	×
LPLRFa (SEQ ID No: 15) (Peptide Ken)		×	×
SRAHQHSMEIRTPDINPTWYTGRGIRPVGRFa (SEQ ID No: 16) (PCT/JP96/03821)	bPrRP31	△	
TPDINPAWYAGRGIRPVGRFa (SEQ ID No: 17) (PCT/JP96/03821)	rPrRP20	△	×
SPEIDPFWYVGRGVRPIGRFa (SEQ ID No: 18) (M. Fujimoto et al., Biochem. Biophys. Res. Commun. 242: 436-440 1998)	cRFa	△	×
SGQSWRPQGRFa (SEQ ID No: 19) (Bachem)	ACEP-1	△	×

Table 2

LSSFVRIa (SEQ ID No: 20) (Bachem)		△	×
ARPGYLAFPRMa (SEQ ID No: 21) (Bachem)	SCPA	×	×
MNYLAFPRMa (SEQ ID No: 22) (Bachem)	SCPB	×	×
YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYa (SEQ ID No: 23) (Peptide Ken)	neuropeptide Y	×	×
HKTDSFVGLMa (SEQ ID No: 24) (Peptide Ken)	neurokinin A	×	×
pEPLPDCCRQKTCSCRLYELLHGAGNHAAGILTLa (SEQ ID No: 25) (Peptide Ken)	orexin A	×	
RSGPPGLQGRLQRLLQASGNHAAGILTMa (SEQ ID No: 26) (Peptide Ken)	orexin B	×	